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KJETIL TASKÉN et al

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Sir:

Submitted herewith is a certified copy of the original priority document (NO 971997) on which claim to priority was made under 35 U.S.C. § 119. The Examiner is respectfully requested to acknowledge receipt of said priority document.

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The present invention relates to increasing the T cell proliferation in patients suffering from HIV and/or common variable immunodeficiency by modifying the cAMP/PKA induced inhibition of T cell proliferation.

The immune system of mammals has evolved different strategies to defend the organism against the variety of potentially infectious agents. The ability to acquire specific and anamnestic responses against intruders features the adaptive immune system. Main players in the adaptive immune system are B and T lymphocytes, and the specific recognition of antigen by these cells is mediated by receptors with some degree of structural similarity, yet functionally very different. The different receptor specificities are made possible through somatic rearrangement of a limited number of genes and are clonally distributed. The main strategy of this system is to generate a nearly unlimited number of specificities to cover the recognition of almost any foreign antigen. Immunological memory is partly a result of clonal expansion of subsets of T and B cells reacting with a particular antigen, and enables the organism to respond more quickly at the second encounter with the same antigen.

Cell proliferation is used as a parameter for immune activation. According to the clonal selection theory, exposure to antigen leads to activation of individual B and T cell clones with corresponding receptor-specificities. However, the number of cells with affinity for a certain antigen is a small fraction of the total number of cells (~0,001%). It is therefore crucial that the activated cells are capable of proliferation (clonal expansion) in order to generate an adequate immune response. Thus, proliferation is a very important parameter characterizing lymphocyte function and capability of immune activation. In in vitro experiments, it is possible to activate the total population of isolated T lymphocytes by using antibodies directed against the antigen receptor complex (TCR/CD3). This will mimic the in vivo situation when T cells are immunoactivated to clonal expansion through the antigen receptor. It is known that T cell proliferation is inhibited through the cAMP signalling pathway.

Cyclic AMP-dependent protein kinase (PKA) is an enzyme present in all cells. Hormones and neurotransmitters binding to specific receptors stimulate the generation of the second messenger 3', 5'-cyclic adenosine monophosphate (cAMP). Cyclic AMP is one of the most common and versatile second messengers and exerts its action by binding to and activate PKA. PKA is a serine/threonine protein kinase which phosphorylate a number of different proteins within a cell, and thereby regulate their activity. It is known that PKA regulates a vast variety of cellular processes such as metabolism, proliferation, differentiation and regulation of gene transcription. PKA is made up of four different subunits, a regulatory (R) subunit dimer and two catalytic (C) subunits. Furthermore, two main isoforms of PKA, PKA

type I and PKA type II (PKAI and PKAII, respectively) have been described. PKAI and PKAII can be distinguished due to their R subunits, designated RI and RII. Isoforms of RI and RII are called RIα, RIβ, RIIα, RIIβ. Moreover, the C subunits also exist as isoforms referred to as Cα, Cβ and Cγ. The different subunits may form multiple forms of PKA (isozymes) which display different localization within a cell as well as different functions.

PKA is a key negative regulator of lymphocyte function. The present inventors and others have shown that cAMP inhibits T lymphocyte proliferation induced through the T cell antigen receptor/CD3 complex (TCR/CD3). We have shown that T cells express both PKAI and PKAII. However, only the selective activation of PKAI is sufficient to mediate the inhibitory effect of cAMP. In addition we have demonstrated that PKAI, but not PKAII, redistribute to and colocalize and inhibit signalling through antigen receptors on B cells and natural killer cells and regulate mitogenic responses in B cells and acute cytotoxic responses in NK cells. Thus, PKAI serve as a key negative regulator of lymphocyte functions as mitogenic and cytotoxic responses initated through antigen receptors.

HIV and Common variable immunodeficiency (CVI). Both primary and secondary immunodeficiencies cause an increased incidence of opportunistic infections and cancer, and are increasing causes of morbidity and mortality in all parts of the world. Human immunodeficiency virus (HIV) causes a chronic infection leading to severe dysfunction of the immune system with markedly increased incidence of a large number of infections and certain forms of malignancies (e.g. lymphoma and Kaposis' sarcoma). In many communities in USA, HIV infection is the leading cause of death among "young" adults. In the developing world this problem is even larger. Next to immunoglobulin (Ig) A deficiency, common variable immunodeficiency (CVI) is the most frequent type of primary immunodeficiency. This form of primary hypogammaglobulinaemia is characterized by onset of immunodeficiency after the first two years of life, by severely decreased serum IgG level and recurrent bacterial infections, particularly in the respiratory tract.

Cellular defects in immunodeficiencies. T cell dysfunction is the immunologic hallmark of HIV infection. Defective lymphocyte cytokine production and impaired proliferative response on stimulation are early signs of immunodeficiency in these patients, manifested even before a decline in CD4+ lymphocyte counts is observed. B cell dysfunction with impaired antibody synthesis is the major immunologic characteristic of CVI patients. However, the immunologic abnormalities in CVI are not restricted to B cells, but often also involve T cell dysfunction, e.g. impaired proliferative response on stimulation. The B cells in CVI patients are not necessarily intrinsically defective, and impaired T cell "help" may be of importance for the B cell defect in these patients. T cell dysfunction may also be of

importance for certain clinical manifestations in these patients not necessarily related to defective antibody production, e.g. increased incidence of granulomata and malignancies.

Current therapies. Antiretroviral therapy is the main component in the treatment of HIV-infected patients. However, although potent antiretroviral combination therapy may markedly increase the CD4+ and CD8+ lymphocyte counts in HIV-infected patients, impaired T cell function seems to persist. Thus, there is a need for immunomodulating agents in addition to antiretroviral therapy in these patients. Immunoglobulin substitution is the main component in the treatment of CVI patients. However, this substitution therapy does not restore the defective T and B cell function. Furthermore, in some clinical complications, e.g. noncaseating granulomata and persistent viral infections, there is need for therapy which more directly may enhance T cell function.

Therapeutic potential of novel drugs targeting T cell dysfunction. Although impaired T cell function is a well recognized immunologic feature of both HIV infection and CVI, the exact molecular mechanism for this T cell impairment is unknown. Therapeutical modalities directed against such intracellular defects may be of major importance in the treatment of these patients, and may have the potential to restore important immunologic defects in HIV-infected patients and in patients with CVI.

Hofmann et al. (Aids, Vol 7; 659-664, 1993) has demonstrated that HIV-seropositive individuals without AIDS showed significant increase in intracellular cAMP levels and PKA activity in peripheral blood mononuclear cells (PBMC) from HIV-seropositive subjects. Examination of T cells was reported as data not shown and did not reach significance because of larger variability, probably induced by the T cell purification method. Their study further indicated that adenosine analogues such as 2',5' dideoxyadenosine (ddAdo) reduced cellular cAMP levels in PBMC and increased the cell proliferation. This effect was, however, concentration-dependent such that concentrations in the range of 6 ng/ml were effective and higher concentrations were suppressive or did not further inhibit cAMP levels. It was not demonstrated similar effects in the T cells, neither a simple concentration/response relationship.

Chu-Chung YS, Genieser H and Jastorff, B (WO 93/21929-A) have shown treatment applied to cancer cells by antagonising cAMP-dependent protein kinases, by using phosphorothoate derivatives of cAMP.

In summary it is not known in the prior art the role of the cAMP level in T cells regarding cell proliferation and immune response during physiological conditions. Furthermore, it is

not known the role of the protein kinase isoenzymes regarding T cell functions during physiological conditions and if cAMP/PKAI has immunomodulating activity. Although the results of Hofman et al (vide supra) suggested increased cAMP level in mononuclear cells, it was not presented documentation supporting a similar condition in T cells. It is thus not known if the cAMP level is increased in purified T cells and negatively selected (not activated) T cells from HIV+ subjects, and nothing regarding the cAMP/PKA pathway in patient suffering from CVI.

It is therefore the object of the present invention to specifically increase the T cells proliferation in HIV and common variable immunodeficiency patients by using suitable compounds interfering with the cAMP/PKA pathway in the T cells.

This object is obtained by the present invention characterized by the enclosed claims.

Disruption of effects mediated by cAMP dependent protein kinase can be performed in the following ways:

Kinase inhibitors. Effects of cAMP mediated through PKA can be disrupted by inhibition of the activated kinase by the highly specific protein kinase inhibitor, PKI, an endogenous 75-amino acid protein. However, this is not useful for living cells or whole organisms as the protein can not be introduced in the cell, but stable transfectants overexpressing PKI can be used. Other diffusible inhibitors of the activated kinase with quite poor specificity and/or affinity are H8, H89 and KT5720.

PKA isozyme-specific cAMP antagonists. The activity of PKA is specifically regulated through the R subunit by cAMP in vivo. Chemically modified, diffusible cAMP analogs can be used to manipulate intracellular levels of cAMP. Thio-substituted analogs where the phosphorous in the cyclic phosphate of cAMP has been exchanged with sulfur, produces a compound resistant to breakdown by phosphodiesterases. Furthermore, thio-substituted analogs can have two forms; the sulfur may be in an equatorial or axial position versus the adenine ring. Whereas the axial diastereomer (Sp-cAMP-phosphorothioate, Sp-cAMPS and derivatives) serves as a cAMP agonist, the equatorial diastereomer (Rp-cAMP-phosphorothioate, Rp-cAMPS and derivatives) binds competetively to the cAMP binding sites of the R subunits, but does not activate the enzyme. A more detailed characterization demonstrated that Rp-cAMPS works as a full antagonist of PKAI isozymes only ($RI\alpha_2C_2$, $RI\beta_2C_2$) and as a partial agonist of PKAII isozymes ($RII\alpha_2C_2$, $RII\beta_2C_2$). Examples of diffusible derivatives of Rp-cAMPS useful in living cells are Rp-8-Br-cAMPS, Rp-8-Br-

MB-cAMPS, Rp-MB-cAMPS, Rp-8-Cl-cAMPS, Rp-8-(4-chlorophenylthio)-cAMPS, Rp-piperidino-cAMPS.

<u>Gene function knock out strategies.</u> Effects mediated by specific isozymes of PKA can be disrupted by inhibition of synthesis of subunits of that enzyme complex. This can be accomplished by the use of sequence-specific antisense oligonucleotides hybridizing to mRNA and blocking translation for subunits of PKA. Another strategy is to use hammerhead ribozymes specifically recognizing and cleaving mRNA for subunits of PKA to inhibit synthesis of these subunits.

<u>Ribozymes.</u> Ribozymes are RNA molecules which catalyse the cleavage and formation of phosphodiester bonds. The discovery of short oligoribonucleotides with endoribonuclease activity has provided researchers with an important tool to block expression of specific genes. The hammerhead domain is a good candidate for incorporation in gene-specific antisense transcripts and induces a enzymatic cleavage at a targeted GUC-sequence in the mRNA and disrupts subsequent translation of the mRNA to protein. Ribozyme can be transfected into cells as RNA or introduced as minigenes from which the ribozyme is transcribed intracellularly under control of a promotor. Furthermore, ribozymes can be chemically modified by addition of alkylgroups in 2'-position of the ribose moiety to stabilize the RNA and prolong intracellular effects.

<u>Sequence-specific antisense oligonucleotides.</u> Oligonucleotides antisense to specific mRNAs will bind to RNA in a DNA/RNA heteroduplex and inhibit translation of mRNA to protein by blocking the movement and reading by the translation machinery. Thio-substituted analogs are more stable to degradation and can be transfected to block translation of specific genes.

<u>Disruption of anchoring.</u> Specific cAMP-mediated effects at defined subcellular loci have been shown to be dependent on anchoring of PKA type II via hydrophobic interaction with a amphipatic helix domain in A kinase anchoring proteins (AKAPs) in close proximity to substrate at that subcellular location. Disruption of anchoring by 22-amino acid competition peptides to the interaction domain introduced by liposome-mediated peptide transfer, abolishes isozyme-specific effects. No similar effects has as of yet been shown for PKA type I. Example of a AKAP-competitor peptide:

Ht31 (439-515): 22-mer aminoter-Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Glu-Val-Lys-Ala-Ala-Tyr-carboxyter.

According to the invention disruption of the cAMP-induced inhibition of T cell immune responses can be obtained by abolishing PKA type I/RIα. Hammerhead ribozymes to PKA type I will be synthesised and tested in vitro and in vivo by transfection of cell lines, followed by transfection of peripheral blood T cells. Functional consequences of ribozyme treatment will be measured as anti-CD3 induced proliferation and IL-2 production.

Example of hammerhead ribozyme designed to knock-out the RIα subunit of PKA type I:

GUACUGCCACUGAUGAGUCCGUGAGGACGAAACUCCAUG

example of antisense oligo: to RIα:

GTACTGCCAGACTCCATG

Disruption of anchoring has been shown for PKA type II, but not for PKA type I. By cloning PKA type I anchoring proteins we will use far-western technique employing RIα as the probe on screenings of blood cell expression libraries. Furthermore, we are employing a yeast two-hybrid system to detect interacting proteins. The binding domain will be characterized by deletional mapping and mutation analysis. Competitor peptides will be designed.

According to the present invention cAMP antagonists, and/or suitable hammerhead ribozyme and/or competitive achoring peptides are used as a therapeutic means to increase T cell proliferation in patients with HIV and CVI in vitro, ex vivo and in vivo.

The active chemical compound(s) will be administered to a patient in need of treatment via all systemic administration routes known in the art. Consequently the medicament according to the invention is composed of active chemical ingredients, adjuvants, pharmaceutically acceptable fillers and comprises tablets, suppositories, injection fluids, infusion fluids and powder for production of any choice of administration form.

The requirements for a compound which is directed to modify the T cell cAMP-PKA pathway are at least diffusibility into the T cells and resistance to breakdown by phosphodiesterases. The present invention have demonstrated that a derivative of Rp-cAMP, such as Rp-8-Br-cAMPS in vitro, specifically increased the proliferation of purified T cells from HIV positive patients and in patients with common variable immunodeficiency. It was an especially surprising finding that when the anti-CD3-induced and greatly reduced proliferation of T cells from a HIV patient was investigated, the inventors observed that not

only did the use of the antagonist Rp-8-Br-cAMPS reverse the effect of the complementary cAMP agonist, but further increased the proliferation above the levels in untreated cells. Within the concentration range used (0-1000 mM) the T cell proliferation, expressed by [^{3}H]-thymidine incorporation, correlated with the concentration. No increased T cell proliferation by using the cAMP antagonist was demonstrated in normal subjects.

The invention is in the following described in further detail by examples and figures, wherein

Fig. 1. (A) Levels of endogenous cAMP were examined in peripheral blood CD3+ T cells from normal healthy blood donors (n=10) and HIV-infected patients (n=9). CD3+ T cells were isolated at 4 C by negative selection. Human peripheral blood CD3+ T cells were purified by negative selection from 50 ml of heparin-treated blood from normal healthy donors (Ullevaal University Hospital Blood Center, Oslo, Norway) or patients. Briefly, peripheral blood mononuclear cells were isolated by density gradient (Lymphoprep, NycoMed, Oslo, Norway) centrifugation followed by negative selection using monodisperse magnetic beads directly coated with antibodies to CD14 and CD19 and rat anti-mouse IgG beads coated with antibodies to CD56 and a magnet. Magnetic beads were all from Dynal (Oslo, Norway, cat. no. 111.12, 111.04, and 110.11, respectively) whereas anti-CD56 antibody was from Pharmingen (San Diego, CA, cat. no. 31660.d). All steps were performed at 4°C. Cell suspensions were routinely screened by flow cytometry using fluorescent antibodies and a FacScan (Becton-Dickinson, San Diego, CA) and shown to consist of more than 90 % CD3+ and low levels of CD14+ (<2%), CD19+ (<2%) and CD56+ (<5%) cells. CD4+ lymphocyte count and viral load were determined for all patients (Tab. 1). Triplicate samples (2 x 10^6 cells) were harvested, followed by subsequent extraction of cAMP and analysis of intracellular cAMP content as described elsewhere (21). Median values (horisontal line) and single patient data (open circles) are shown. Basal levels of cAMP were shown to be stable at 4°C both in crude peripheral blood mononuclear cells and CD3+ T cells for more than 120 min (the interval required for purification of CD3+ T cells). * denotes $p<0.05$ (B) TCR/CD3 stimulated proliferation of peripheral blood CD3+ T cells from normal healthy blood donors and HIV-infected patients was assessed as [^{3}H]-thymidine incorporation in the presence of increasing concentrations of 8-(4-chlorophenylthio)cAMP (8-CPT-cAMP). Normalized levels of proliferation of T cells from a healthy blood donor (open circles) and a representative HIV-infected patient (solid circles) are shown (IC_{50} values 6.11 microM vs. 1.78 microM). Note: Left-shift of IC_{50} (arrow) and altered curve slope. The maximal levels of proliferation were drastically decreased in T cells from HIV-infected patients (see Table 1). Proliferation assays were performed by incubation of 0.75 X 10^6 CD3+ T cells/ml in a 100 microl volume in flat-bottom 96-well microtiter

plates. Activation was achieved by subsequent addition of monodisperse magnetic beads coated with sheep anti-mouse IgG (Dynal, cat. no. 110.02) at a cell:bead ratio of 1:1 followed by addition of anti-CD3 (clone $SpvT_3b$) at a final dilution of 1:125,000 for the experiments shown. The optimal concentration of antibody was titrated carefully in the initial setup and parallel experiments at several different dilutions of antibody were always performed. Proliferation was analysed by incubating cells for 72 hours during which [^{3}H]-thymidine was included for the last 16 hours. Cells were washed and harvested onto filters using a Scatron harvester (Suffolk, UK) and subsequently analyzed by beta-scintillation counting. cAMP analogs, when used, were added 30 min prior to activation by addition of anti-CD3 antibodies. 8-CPT-cAMP was from Sigma (St. Louis, MO) and Sp- and Rp-8-Br-cAMPS were from BioLog Life Science Company (Bremen, Germany) and were all dissolved to concentrations of 4 to 10 mM in PBS and concentrations calculated using the extinction coefficients given by the manufacturer. Curve-fit analyses were performed and IC_{50} values calculated (see Tab. 1 for statistics and single patient data). Curve fit analyses were performed using Sigma Plot (Jandel Corporation, Erkrath, Germany) and statistics were analysed using Statistica (Statsoft Inc., Tulsa, OK).

Fig. 2. Inhibition of TCR/CD3 stimulated T cell proliferation by the cAMP agonist Sp-8-Bromo-cAMP-phosphorothioate (Sp-8-Br-cAMPS) and reversal of inhibition by the complementary cAMP antagonist Rp-8-Bromo-cAMP-phosphorothioate (Rp-8-Br-cAMPS), was assessed in normal healthy blood donors (A) and HIV-infected patients (C). The effect of increasing doses of cAMP antagonist on TCR/CD3 stimulated proliferation of CD3+ T cells isolated from normal blood donors (B) and HIV patients (D) was examined separately in the same experiments. (A) and (B) show proliferation of CD3+ T cells from 3 healthy blood donors that were pooled and purified. (C) and (D): Proliferation of T cells from one patient with symptomatic HIV infection. Mean values of triplicate determinations ± SD are shown. See Table 1 for summarized patient data (n=18). Note: Scaling differs in upper versus lower panels.

Fig. 3. (A) TCR/CD3 stimulated proliferation of peripheral blood CD3+ T cells from a normal healthy blood donor (open circles) and a patient with common variable immunodeficiency (closed circles) in the presence of increasing concentrations og 8-CPTcAMP. Curve fit analysis was performed and IC_{50} values calculated. (B) The effect of cAMP antagonist (Rp-8-Br-cAMPS) on TCR/CD3 stimulated proliferation of CD3+ T cells from a representative patient with common variable immunodeficiency. Mean values of triplicate determinations ± SD are shown. Note: Left-shift of IC_{50} (arrow) and altered curve slope in (A). The basal level of proliferation in T cells from the patient with common

variable immunodeficiency was markedly decreased. Treatment with Rp-8-Br-cAMPs increased proliferation 3.2-fold (B).

Examples

Example 1

Cyclic AMP completely abolishes T cell proliferation induced through TCR/CD3 as well as early tyrosine phosphorylation following engagement of the antigen receptor (1-3). We have previously shown that type I cAMP-dependent protein kinase (PKA) is necessary and sufficient for mediating inhibition of TCR/CD3-induced proliferation and that PKA type I redistributes to and colocalizes with the antigen receptor following activation and capping of T cells (2,4). This serves to establish PKA type I as an acute negative modulator of T cell antigen responses and clonal expansion. Furthermore, activation of T cell proliferation by phorbol ester in combination with calcium ionophore renders the T cell insensitive to the inhibitory action of cAMP indicating a target for PKA type I phosphorylation more proximal to the antigen receptor than the down-stream activation of PKC (3).

T cell dysfunction is an early event in the course of HIV infection and a major factor in the development of severe immunodeficiency. However, the molecular mechanisms by which HIV-infection impairs T cell function have not been revealed. Several recent publications have provided indications that HIV and HIV-derived peptides may increase cAMP levels in vitro and a single report indicates that cAMP is elevated in crude peripheral blood mononuclear cells from HIV-infected patients, containing a mixture of B, T and NK cells as well as the prostaglandin-producing monocytes (5-7). Furthermore, cAMP treatment increased HIV reverse transcriptase activity 5 to 10-fold in a cultured T cell line (8). Together this may serve to establish a circulus vitiosus in the HIV-infected T cell. For this reason, we examined the levels of endogenous cAMP and the sensitivity for cAMP/PKA type I-mediated inhibition of TCR/CD3-induced proliferation in highly purified CD3+ T lymphocytes from HIV-infected patients.

In negatively selected T cells from nine consequtive HIV-infected patients (independent of clinical status) the endogenous levels of cAMP were significantly elevated compared to the levels in CD3+ T cells concomitantly isolated from 10 HIV-seronegative blood donors (476 vs. 275 fmol/10^6 cells, $p<0.05$, see Fig. 1A). Furthermore, the effect of cAMP agonist on TCR/CD3-induced proliferation was investigated in 19 individual HIV-infected patients and 8 seronegative controls (Table 1 and Table 2). The patients were classified in two groups, one group with asympthomatic and the other with sympthomatic HIV-infection (AIDS and

non-AIDS) (Table 1), according to (9). T cells from HIV-infected patients revealed a highly significant increase in sensitivity to inhibition of cell proliferation by exogenously added 8-CPT-cAMP (Fig. 1B and Tabels 1 and 2, $p<0.001$, $n=18$). Moreover, when the maximal proliferation rates of T cells from HIV-infected patients and that of seronegative T cells were normalized to 100% (Fig. 1B and (10)), it was evident that in addition to a distinct left-shifted cAMP-inhibition curve, the slopes of the curves were significantly different (Hill coefficients of 1.19 (1.18-1.38) for T cells from HIV-seropositive individuals versus 1.59 (1.52-1.77) for normal T cells, Tab. 1, $p<0.01$, $n=18$). The increased sensitivity to inhibition by cAMP analog suggests a contribution from elevated endogenous cAMP in priming cAMP binding site A of PKA type I with subsequent increase in the affinity of the B site for the exogenously added cAMP analog. The shift in curve slope from a cooperative, two-ligand site binding situation to an apparent non-cooperative inhibition curve by 8-CPT-cAMP also indicates A-site occupancy by endogenous cAMP.

Table 1

	CD4+lympocyte count	HIV-RNA (24)	α-CD3-induced proliferation	Inhibition of proliferation by 8-CPT-cAMP	Inhibition of proliferation by 8-CPT-cAMP	Increase in proliferation by Rp-8-Br-cAMPS
	Cells/microL	Copies/ml plasma	$[^3H]$-thymidine incorporation (cpm)	IC_{50} (μM)[a]	Hill coefficient	Fold increase compared to untreated
	660 (520-980)[b]	n.d.	132484 (121181-138453)	4.59 (4.04-5.82)	1.59 (1.40-1.81)	1.01 (0.93-1.14)
ic	154 (115-203)	7850 (200-48400)	54558 (35995-77164)***	2.26 (1.96-2.64)**	1.18 (1.09-1.36)**	1.50 (1.29-1.73)**
	42 (10-112)	69300 (13700-333300)	26817 (16745-40035)***	1.87 (1.69-2.30)***	1.19 (1.13-1.40)*	1.62 (1.48-2.00)***

Data are presented as median values and a 25 to 75 % range is indicated in parenthesis (normal blood donors: n=8, asympthomatic and sympthomatic HIV-infection: n=8 and n=10, respectively). Anti-CD3-induced T cell proliferation, inhibition of proliferation by 8-CPT-cAMP (IC_{50} and hill coefficient) and increase in proliferation following treatment with Rp-8-Br-cAMPS in normal versus HIV-infected CD3+ T cells were analysed by Mann-Whitney U test. Significance: * denotes $p<0.05$, ** denotes $p<0.01$ and *** denotes $p<0.001$. [a]IC_{50} denotes the concentration of cAMP analog necessary to produce a half-maximal inhibition of CD3-induced T cell proliferation. [b] Range of CD4+lymphocyte count in 21 blood donors. n.d.: not done.

Table 2

Patients	Clinical status[a]	α CD3-induced proliferation [^{3}H]-thymidine incorporation (cpm)	Inhibition of proliferation by 8-CPT-cAMP IC_{50} (μM)	Increase in proliferation by Rp-8-Br-cAMPS[b] Fold increase compared to untreated
#1	s	11838	0.90	2.81
#2	s	13150	1.87	2.02
#3	s	16745	1.57	2.73
#4	s	17981	1.82	1.48
#5	s	21430	1.69	1.77
#6	a	29957	2.15	1.96
#7	s	32203	3.13	1.50
#8	a	33887	2.63	1.79
#9	s	34857	n.d.	1.74
#10	a	38102	1.78	1.30
#11	s	40035	2.36	1.49
#12	s	45686	2.28	1.43
#13	s	50051	2.03	1.46
#14	a	52195	2.14	1.67
#15	a	56920	2.36	1.60
#16	a	62248	4.04	1.27
#17	s[c]	75380	1.86	1.77
#18	a	92080	2.66	1.39
#19	a	98644	1.55	1.16

[a]Clinical status: a: asymptomatic HIV infection, s: symptomatic HIV infection (AIDS or non-AIDS). [b]Patients were ordered according to alpha-CD3-induced proliferative response. [c]Patient with AIDS that received treatment with HIV-protease inhibitor (Indinavir). The data from this patient were therefore excluded from the groups in Tab. 1. n.d.: not done.

In order to further assess the specificity of the inhibition of TCR/CD3-induced T cell proliferation, we used a sulfur-substituted cAMP analog (Rp-8-Br-cAMPS) working as a full antagonist for PKA type I (11). Figure 2A shows that in T cells from normal blood donors, TCR/CD3-stimulated proliferation was inhibited by a cAMP agonist (Sp-8-Br-cAMPS). This effect was almost completely reversed by increasing concentrations of the complementary antagonist (Rp-8-Br-cAMPS). However, antagonist alone did not alter proliferation of normal T cells (Fig. 2B). In contrast, when the TCR/CD3-induced proliferation of T cells from a HIV-infected patient was investigated, we observed that not only did the use of the antagonist (Rp-8-Br-cAMPS) reverse the effect of the complementary agonist, but further increased the proliferation above the levels in untreated cells (Fig. 2C). When the effect of the cAMP antagonist alone was assessed in T cells from HIV-infected patients, we observed a concentration-dependent increase in TCR/CD3-induced proliferation that was more than 2-fold at higher concentrations (Fig. 2D). The degree of increased proliferation following treatment with cAMP antagonist was inversely correlated with the level of TCR/CD3-induced proliferation in the absence of antagonist ($p<0.001$, $R=0.70$, $n=19$, Tab. 1), i.e. T cells responding poorly to TCR/CD3 stimulation benefited most from cAMP antagonist. The stimulatory effect of the cAMP antagonist was not saturated even at the highest concentrations used (Fig. 2D and (12)). This indicates that the solubility of the compound, affinity, or availability to cells may be a limiting factor for the effect observed. Thus, a more permeable and potent PKA type I antagonist, when available, may further improve TCR/CD3-induced proliferation of T cells from HIV-infected patients.

The above results suggest that increased activation of TCR/CD3-associated PKA type I contributes to the dysfunction of T cells from HIV-infected patients. Elevated levels of cAMP in T cells in HIV-infected patients may reflect effects of HIV-derived peptides or may partly be caused by dysregulated levels of cytokines and other signalling substances. Common variable immunodeficiency (CVI) is a heterogeneous group of severe immunodeficiencies involving impaired B-cell function as well as, in some patients, T cell defects (reviewed in (10,11)). The pathogenetic mechanisms of CVI are not fully understood, but the T cell dysfunction that involves impaired TCR/CD3-induced proliferation and IL-2 production, is to some extent similar to that of HIV-infection (12). For this reason we screened several patients with CVI for impaired T cell function and investigated the effect of cAMP agonist and antagonist in a patient with CVI involving T cell dysfunction. Our results demonstrate a greatly reduced TCR/CD3-stimulated proliferation. Furthermore, inhibition of proliferation by 8-CPT-cAMP was clearly different from that in T cells from normal blood donors (IC_{50} of 2.26 vs. 4.66 microM) and the slope of the cAMP inhibition curve was again altered (Hill coefficients of 1.26 vs. 1.59). This

indicates that chronically elevated levels of endogenous cAMP inhibits mitogenic responses in T cells from this patient. Finally, treatment with the PKAI-selective antagonist increased TCR/CD3-induced proliferation more than 3-fold.

The results described here demonstrate elevated levels of cAMP in T cells from HIV-infected patients. Interestingly, increased cAMP sensitivity, indicating elevated cAMP levels, was observed also in T cells from a patient with CVI that has a T cell dysfunction of a different origin from that of HIV-infection. Furthermore, use of a cAMP antagonist preferentially acting on PKA type I greatly improves the immune response of T cells from these patients as indicated by the proliferative response following TCR/CD3-induced activation. Levels of PKA type I and II were unaltered in T cells from HIV-infected patients compared to normal controls by immunoblot analysis. Previous observations demonstrate that PKA type I not only colocalizes with antigen receptor in T cells, but localizes similarly in B cells and completely abolishes mitogenic responses in both T and B cells and cytotoxic function of NK cells (2,4,13-16). This together with the observations that triggering of the TCR/CD3 complex leads to production of cAMP (2,8,17) has previously lead us to hypothesize that the normal T cell immune responsiveness is negatively modulated by cAMP through PKA type I and that PKA activation following TCR triggering is a negative feedback mechanism.This is further supported by the recent observations that PKA type I is impaired in T cells from patients with systemic lupus erythematosus (18,19) suggesting that cAMP/PKA type I dysfunction may lead to overshoot of immune cell responses thus contributing to the pathogenesis of this autoimmune disease. Vice versa, with the observations made here it appears that elevated levels of cAMP in T cells from immunodeficient patients shifts the equilibrium in the opposite direction and produce a situation where constant inhibition through PKA type I significantly impairs the immune responsiveness of T cells in vitro. Future studies addressing mechanisms that elevate cAMP in T cells from HIV-infected patients as well as in patients with common variable immunodeficiency will be of great interest and may lead us to understand the pathogenetic impact of PKA type I dysregulation in immunodeficiencies in vivo. Further, treatment regimens which counteract the activation of PKA type I, may be a supplement to therapy for patients with HIV-infection as well as other groups of immunodeficiencies still to be defined.

Example 2

cAMP antagonist improves T cell immune responses in immunodeficient T cells from a subgroup of patients with common variable immunodeficiency (CVI)

Purified T cells from a group of 21 patients with the diagnosis "common variable immunodeficincy" (CVI) were examined for proliferative response following triggering of the T cell receptor/CD3 complex (TCR/CD3). The effect on the proliferation of a single dose of a cAMP-antagonist (Rp-8-Br-cAMPS) preferentially acting on PKA type I, was assessed within the same material. A subgroup of 7 patients was defined as having impaired T cell immune response and improved T cell proliferation in the presence of cAMP-antagonist. A detailed examination revealed that TCR/CD3-induced T cell proliferation in T cells from these patients was significantly reduced compared to the proliferation of T cells from normal blood donors. Furthermore, inhibition by exogenously added cAMP-analog (8-CPT-cAMP) revealed a significantly increased inhibition of T cell proliferation. This suggests increased levels of endogenous cAMP already inhibiting T cell immune functions in T cells from a subgroup of patients with CVI. A concentration-dependent increase [median 1.87-fold, ranging from 1.34 to 3.67-fold (Table 3, 4)] in TCR/CD3-induced T cell proliferation in the presence of cAMP-antagonist (Rp-8-Br-cAMPS) was observed.

Table 3

Clinical status	α-CD3-induced proliferation [^{3}H]-thymidine incorporation (cpm)	Inhibition of proliferation by 8-CPT-cAMP IC_{50} (μM)	Increase in proliferation by Rp-8-Br-cAMPS Fold increase compared to untreated
Normal (n=8)	132484 (121181-138453)	4.60 (4.04-5.82)	1.01 (0.93-1.14)
CVI (n=7)	36740 (26968-78080)	2.98 (2.34-3.36)	1.87 (1.37-2.45)

Table 4

Single patient data:

Patients	α-CD3-induced proliferation [^{3}H]-thymidine incorporation (cpm)	Inhibition of proliferation by 8-CPT-cAMP IC_{50} (μM)	Increase in proliferation by Rp-8-Br-cAMPS Fold increase compared to untreated
#1	109567	2.34	1.37
#2	78080	2.66	1.34
#3	66050	3.16	1.87
#4	36740	3.36	2.10
#5	35224	3.68	1.73
#6	26968	2.26	3.67
#7	4870	2.98	2.45

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CLAIMS

1. Use of cAMP antagonist and/or sequence specific antisense nucleotides and/or suitable hammerhead ribozyme and/or anchoring disruption peptides for production of a pharmaceutical preparation to treat immunosupressive diseases.

2. Use according to claim 1, wherein the cAMP antagonist is selected from the group consisting of Rp-8-Br-cAMPS, Rp-8-Br-Mb-cAMPS, Rb-MB-cAMPS, Rb-8-Cl-cAMPS, Rp-8-(4-chlorophenylthiol)-cAMPS and Rp-8-piperidino-cAMPS.

3. Use according to claims 1 and 2, wherein the cAMP antagonist is Rp-8-Br-cAMPS.

4. Use according to claims 1-3, wherein the immunosuppressive disease is HIV or CVI.

5. Use according to claim 1, wherein the hammerhead ribozyme has the following base sequence; GUACUGCCACUGAUGAGUCCGUGAGGACGAAACUCCAUG.

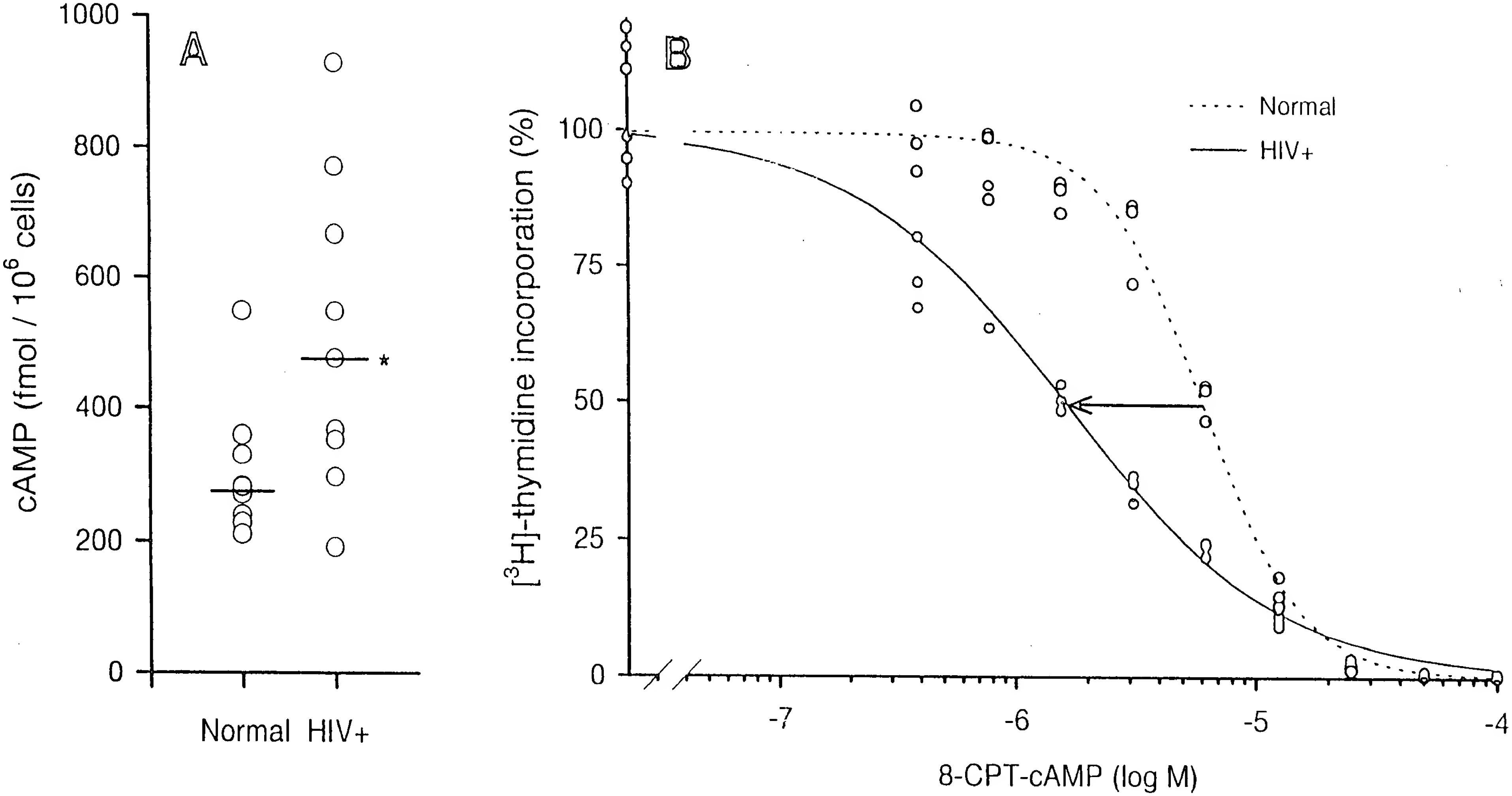
6. Use according to claim 1, wherein the sequence specific antisense nucleotid has the base sequence; GTACTGCCAGACTCCATG.

7. Use according to claim 1, wherein the competitive anchoring disruptive peptide contains at least 22 amino acids.

8. Use according to claim 6, wherein the amino acids are aminoterminal-Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Glu-Val-Lys-Ala-Ala-Tyr-carboxyterminal.

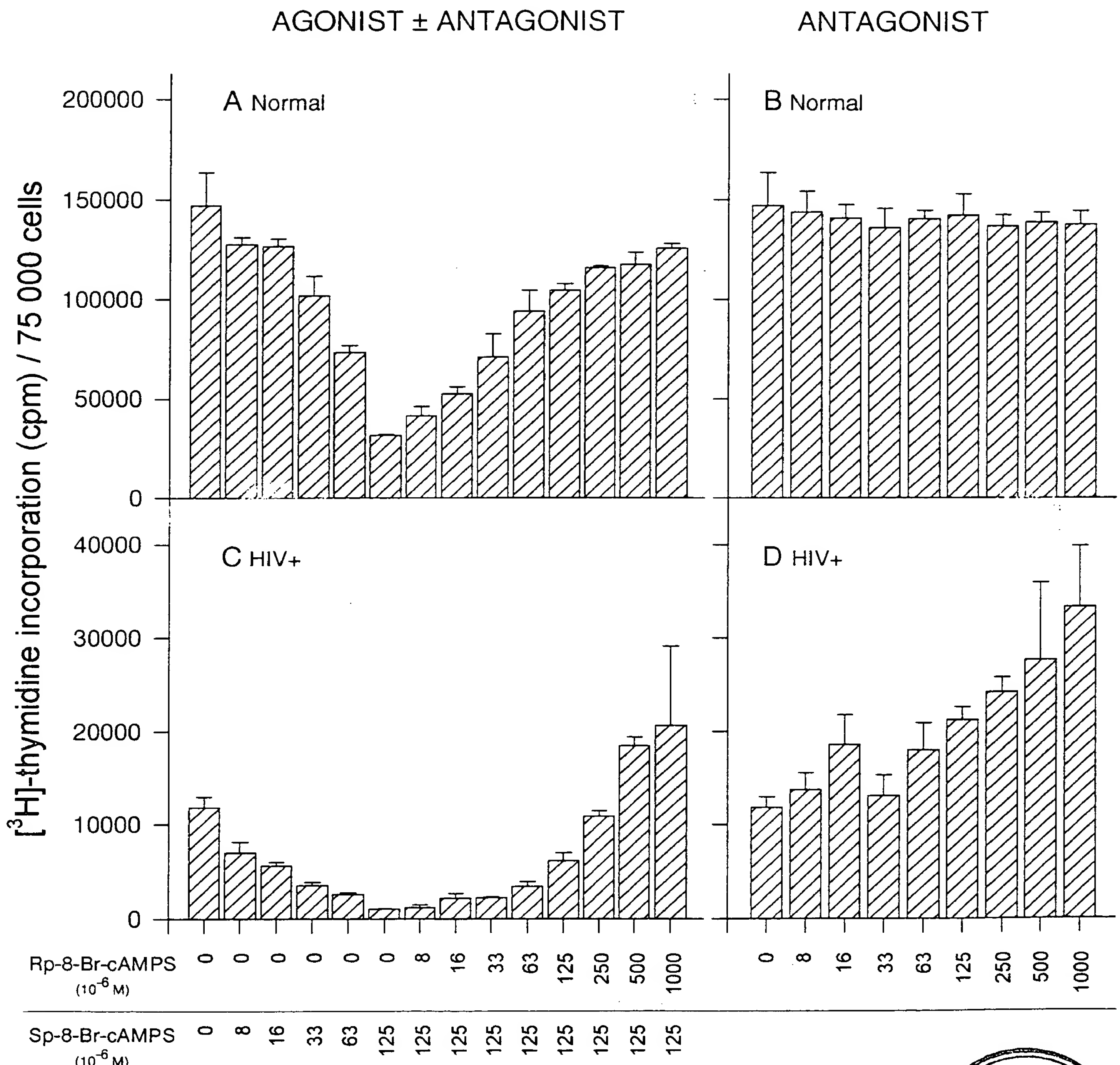
ABSTRACT

It is described use of several compunds to produce a medicament increasing the T cell proliferation in patients with immunosuppresive diseases.



1000
800
600
400
200
0
cAMP (fmol / 10^6 cells)
A
*
Normal HIV+
B
100
75
50
25
0
[3H]-thymidine incorporation (%)
Normal
HIV+
-7
-6
-5
-4
8-CPT-cAMP (log M)

Fig. 1



AGONIST ± ANTAGONIST
ANTAGONIST
A Normal
B Normal
C HIV+
D HIV+
[3H]-thymidine incorporation (cpm) / 75 000 cells
200000
150000
100000
50000
0
40000
30000
20000
10000
0
Rp-8-Br-cAMPS (10-6 M)
0 0 0 0 0 0 8 16 33 63 125 250 500 1000
0 8 16 33 63 125 250 500 1000
Sp-8-Br-cAMPS (10-6 M)
0 8 16 33 63 125 125 125 125 125 125 125 125 125

Fig. 2

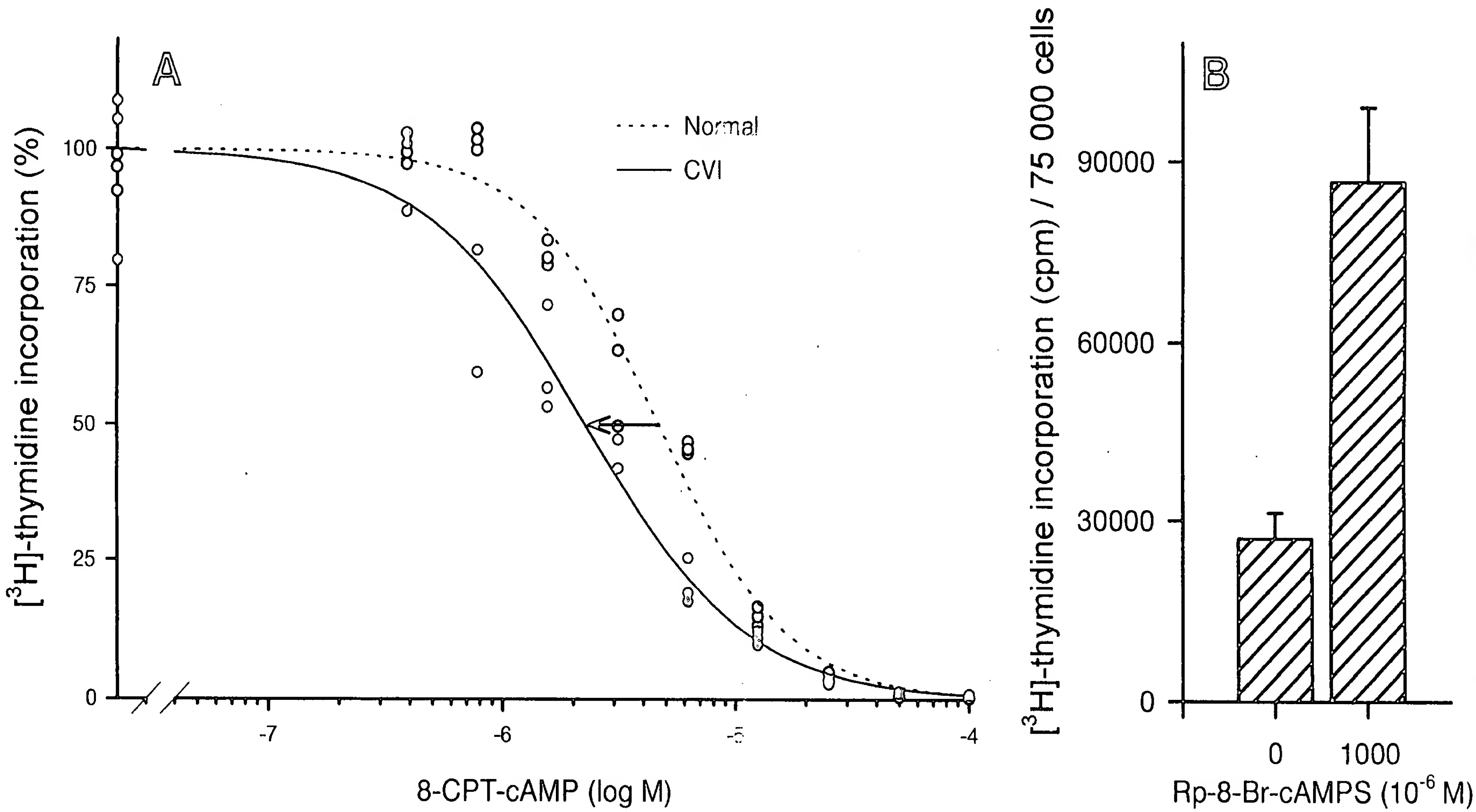

A
B
Normal
CVI
[3H]-thymidine incorporation (%)
100
75
50
25
0
-7
-6
-5
-4
8-CPT-cAMP (log M)
[3H]-thymidine incorporation (cpm) / 75 000 cells
90000
60000
30000
0
0
1000
Rp-8-Br-cAMPS (10-6 M)

Fig. 3